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Abstract

Entry into mitosis is triggered by the activation of Cdk1. This simple reaction rapidly and irreversibly sets the cell up for division. Even though the core step in triggering mitosis is so simple, the regulation of this cellular switch is highly complex, involving a large number of interconnected signalling cascades. We do have a detailed knowledge of most of the components of this network, but only a poor understanding of how they work together to create a precise and robust system that ensures that mitosis is triggered at the right time and in an orderly fashion. In this review we will give an overview of the literature that describes the Cdk1 activation network and then address questions relating to the systems biology of this switch. How is timing of the trigger controlled? How is mitosis insulated from interphase? What determines the sequence of events, following the initial trigger of Cdk1 activation? Which elements ensure robustness in timing and execution of the switch? How has this system been adapted to the high levels of replication stress in cancer cells?

Introduction: The mitotic trigger is an irreversible cellular switch

As cells enter mitosis, a remarkable sequence of events springs into action that appears to proceed with the precision of a finely tuned clock-work mechanism. Centrosomes separate and mature, tightening of the actin cortex causes cell rounding, the chromosomes begin to condense and finally the nuclear envelope breaks down and the mitotic spindle emanates from its poles to capture the sister chromatids [1]. These changes affect every cellular compartment and take place in a brief window of approximately 10-20 minutes. They are driven by the phosphorylation of over 1000 proteins by Cdk1 and its mitotic Cyclin partners Cyclin A and B, as well as by other mitotic kinases such as Plk1, Aurora A and B and NIMA related kinases (NEK) [2,3]. On the flip side, mitotic phosphorylations are removed by Serine/Threonine phosphatases that also appear to be tightly regulated in mitosis [4,5]. Thus, activation of mitotic Cdk1 and inactivation of Cdk1 opposing phosphatases constitute the central elements in this switch system [6]. A complex network of feedback control between Cdk1 and its activators and inhibitors has evolved that ensures a rapid, yet stable induction of the mitotic state [7,8]. Equally important is to ensure that the switch is triggered at the right time. A key feature here is to link progression into mitosis with the completion of DNA replication, but other features such as DNA damage response, metabolism, and size control are also likely to impact on these regulatory mechanisms [9]. Moreover, the rapid activation of Cdk1 has to be translated in a tightly controlled sequence of phosphorylation events that proceeds in a timely and orderly fashion. How timing of Cdk1 dependent phosphorylation is controlled during mitotic entry remains largely unexplored. Finally, a key feature of this regulatory network is its ability to robustly proceed each time a cell divides, despite considerable single cell variation in the diverse signalling events and a noisy extracellular environment. The robust execution of mitotic entry is critical for cellular survival and the maintenance of a stable genome. As with many other cell cycle control events, the G2/M transition appears to be often deregulated in various types of cancer cells. Thus, recent years have seen a growing interest in this area of cell cycle control as a source of potential clinical targets for cancer therapy. Indeed, inhibitors of the Cdk1 regulatory kinases Wee1/Myt1 show promising results in clinical trials [10], while the PP2A regulatory kinase Greatwall is attracting interest as a potential therapeutic target [11].

In this review we will briefly summarise the current state of knowledge on the Cdk1 activation and phosphatase inactivation mechanisms highlighting open question in these areas. Subsequently, we will discuss what is known about the mechanisms that regulate accurate timing and stable establishment of mitosis. Furthermore, we will speculate on how mitotic progression is coordinated by timely establishment of various phosphorylation events, and which design features of this system may be critical for its robust execution. We will conclude this review with a brief outlook on how and why cancer cells might have changed the G2/M switch system, and how this could be further exploited by therapeutic intervention. For other aspects of mitotic entry dynamics, such as spatial

regulation and functions of other mitotic kinases, the reader is referred to excellent recent reviews [7,12-15].

Pulling the trigger: Cdk1 activation

General principles of Cdk1 regulation

Mitosis is initiated by the activation of Cyclin dependent kinase 1 (Cdk1) [16,17]. Activity of this kinase is controlled at several levels. Firstly, the kinase requires a Cyclin partner (in the case of Cdk1, Cyclin A or B) to establish a structural configuration that is permissive for kinase activity [18-20] reviewed by [21]. Synthesis and destruction of mitotic Cyclins are thus the primary regulatory mechanisms that underlie the rise and fall of Cdk1 activity during mitotic entry and exit. Secondly, an activating phosphorylation at a conserved T-loop residue (Thr160 in human Cdk1) is essential for kinase activity [22]. Another Cdk, Cdk7 complexed to Cyclin H, is responsible for this phosphorylation [23,24], which is thought to be constitutive and not employed in signalling responses. The ultimate on and off switch of Cdk1 in interphase is mediated by an inhibitory phosphorylation at Thr14, Tyr15 [25] that is maintained by the Wee1/Myt1 kinases [26-29]. Removal of these inhibitory phosphate groups by the dual specific Cdc25 phosphatases triggers mitotic entry [30,31]. This regulatory mechanism ensures that the steady rise of mitotic Cyclins in interphase is translated in a sharp ultra-sensitive signal response of Cdk1 activity at the onset of mitosis [32]. It is somewhat surprising that Cdk2 cannot take over and induce mitosis in the absence of Cdk1 activity. However, the genetic data regarding this specialised role of Cdk1 are convincing. In mice Cdk2 is only required for male and female meiosis, while Cdk1 is essential for each cell cycle [33-35]. For S-phase progression Cdk2 and Cdk1 act redundantly, but inhibition of Cdk1 by small molecules or chemical genetics results in an arrest in late G2 phase before the initiation of the Cdk1 activation feedback loops [36,37].

Once active, Cdk1:Cyclin complexes cause the major rearrangements that mark mitotic entry by phosphorylating over 1000 proteins. In parallel, other mitotic kinases Aurora A and B, and Plk1 are activated downstream of, or simultaneously with Cdk1 to support mitotic entry by phosphorylating additional mitotic substrates [2,3]. Gavet and Pines [38] have measured the level of Cdk1 activity using a Cdk1 specific FRET probe and correlated the stepwise progression into mitosis with increased activity of Cdk1. These observations support a model whereby mitotic entry is coordinated by various thresholds for Cdk1 activity with early prophase substrates being more susceptible to phosphorylation than substrates at NEBD and in prometaphase. During the first steps of mitosis Cdk1 acts both in complex with Cyclin A and B, but the interplay of these Cdk:Cyclin complexes remains poorly understood. Once the mitotic state is fully established, Cyclin A is degraded in prometaphase prior to Cyclin B in metaphase by the 26S proteasome [39,40]. This is triggered by the Anaphase Promoting Complex/Cyclosome (APC/C), a large multimeric ubiquitin ligase (reviewed by [41]). Cyclin B destruction is delayed by the spindle assembly checkpoint until

all sister-chromatids are aligned at the metaphase plate. Once this is achieved, checkpoint inhibition is relieved and the APC/C triggers the degradation of Cyclin B, promoting mitotic exit [42], reviewed by [43-45].

Cyclins are not the only proteins that can allosterically activate Cdks. Indeed, a separate class of unrelated Cdk activators termed Speedy, or Ringo (Rapid Inducer of G2/M progression in Oocytes) can also bind to and activate Cdk1 [46-48] and homologues of these proteins also play an, as yet, poorly understood role in mitotic regulation in mammalian cells [49,50]. There may well be other "non-Cyclin" allosteric regulators of Cdk1, such as RGC-32 that plays an important role in changing cell cycle control in response to Epstein Barr viral infection [51,52]

Functions of Cyclin A and B in mitosis

The first step in activation of Cdk1 is the binding of their Cyclin partners, Cyclin A and B, that act as allosteric activators [53]. Cyclin A and B levels are controlled by cell cycle specific transcription and translation during S and G2 phase, as well as by induced proteolysis in anaphase and G1 phase by the APC/C. Cyclin A is predominantly nuclear [54] and partners both the S-phase kinase Cdk2, and mitotic Cdk1 where it is active in early mitosis, while Cdk1:Cyclin B is thought to constitute the major M-phase Cdk activity in higher eukaryotic cells (reviewed by [55]). Cyclin B is enriched in the cytoplasm [54], but continuously shuttles in and out of the nucleus, until it translocates to the nucleus shortly before nuclear envelope breakdown (NEBD) [54,56-61]. Work in model organisms such as yeast, Xenopus egg extracts and Drosophila revealed that Cyclins have evolved specific functions and direct the substrate specificity of Cdk1 (reviewed by [55,62]). Conversely, both fission and budding yeast can be engineered to support the progression from S to M-phase in the presence of a single Cdk:Cyclin complex [63-65], supporting a simple quantitative threshold model at the core of cell cycle control. The specific functions of mammalian Cyclin A and B have been analysed genetically in knock out mouse studies and siRNA depletion experiments in human cells. These results proved to be surprisingly contradictory. In mouse embryos, Cyclin B1 is essential to bring cells into mitosis [66,67]. Cyclin A2, the somatic paralogue of the mammalian Cyclin A family, is essential for development, but mouse embryonic fibroblasts can proliferate in the absence of this Cyclin [68,69]. In mammalian cells, Cyclin A2 depletion causes defects both in S-phase progression and in G2 phase [70-75]. Cyclin A has been proposed to constitute the actual trigger of mitotic Cdk1 activation, but the G2 delays or arrest observed in the absence of Cyclin A could be an effect of incomplete replication. Cyclin B1 depletion by siRNA in HeLa or RPE-1 cells has surprisingly mild cell cycle defects. In contrast to the data from mouse knock out studies, human Cyclin B1 and B2 appear to compensate for each other [73,76,77]. Moreover, even co-depletion of Cyclin B1 and B2 is not sufficient to block mitotic entry in HeLa and RPE-1 cells, suggesting that Cyclin A can compensate to some extent for the mitotic entry function of Cyclin B [73]. We have recently revisited this question using degron tags, a more efficient and precise depletion approach compared to siRNA [78]. This study supports the idea that Cyclin A activity in G2 phase is

essential to trigger mitosis. Our results also support the notion that past triggering the Cdk1 activation feedback loops, Cyclin A and B are largely redundant in supporting mitotic entry until prometapahse. Cyclin B is only essentially required to phosphorylate a defined sub-set of mitotic substrates that are involved in metaphase establishment and sister chromatid segregation.

The question remains, what determines the functional divergence of the two mammalian mitotic Cyclins? This functional specialisation could occur at the level of direct substrate interaction as suggested by biochemical studies [79-81]. Specific enrichment of Cdk1 kinase activity at a particular cellular localisation could also be important. Accordingly, Cyclin B is known to be recruited to the kinetochore and has recently been shown to contribute to spindle assembly checkpoint signalling by phosphorylating Mps1 [82,83]. A large subset of the Cyclin B specific substrates that we have identified using degron mediated depletion are indeed associated with the centromere/kinetochore supporting the importance of this targeted localisation of Cyclin B [78]. Nevertheless we also identified Cyclin B specific substrates that are distributed all over the chromosomes such as Ki67, and proteins at the cell cortex. Thus, kinetochore enrichment is unlikely to be the sole functional determinant of Cyclin B in mitosis. Further biochemical and genetic experiments will be necessary to address the precise balance of quantitative versus qualitative functions of Cyclin A and B in human cells. The apparent divergence between mouse and human Cyclin function will also need to be addressed by comparing Cyclin function in somatic cell systems and embryonic stem cell cycles.

Wee1/Myt1 and Cdc25 regulation

The critical biochemical reaction that constitutes the mitotic switch is the removal of the inhibitory phosphates from Thr14 and Tyr15 on Cdk1. The kinases responsible for these phosphorylations are Wee1 and Myt1, with Myt1 phosphorylating both residues and residing in the cytoplasm and Wee1 acting as a specific Tyrosine kinase in the nucleus [26-29,84,85]. Cdk1 activation is then triggered by the removal of these inhibitory phospho-sites. This is brought about by the inactivation of Wee1/Myt1 and activation of the Cdc25 phosphatases (Cdc25A,B and C) that catalyse the dephosphorylation of both Thr14 and Tyr15 in Cdk1 [86,87]. In somatic mammalian cells, Wee1 appears to be the major effector of Cdk1 inhibition, since Myt1 depletion by siRNA does not appear to affect the timing of Cdk1 activation. However, this kinase may contribute to the DNA damage response and to regulation of mitotic Golgi disassembly [88-90]. Mouse genetics suggest that Cdc25B and C can be removed without noticeable effects on viability [91-93], although Cdc25B knock out mice are sterile and oocytes undergo permeant meiotic arrest [93]. Mouse embryos do not develop in the absence of Cdc25A, but the different paralogues appear to compensate for each other in adult tissues and only removal of all three paralogues halts somatic cell proliferation [94,95]. Likewise, CRISPR/Cas9 mediated deletion of Cdc25A in murine embryonic stem cells does not prevent proliferation [96]. This suggests that Wee1 and a single Cdc25 paralogue are sufficient elements for effective G2/M control.

The mechanisms that regulate these enzymes at the G2/M transition are the critical nuts and bolts that make up the mitotic switch system. However, current models of this transition are relatively simplistic in terms of Cdc25 activation and Wee1 inactivation by Cdk1. In fact, these enzymes are regulated in multiple ways. The major control elements for both Wee1 and Cdc25 regulation include activating and inhibitory phosphorylation by various kinases (Chk1, CK1, Cdk1, Cdk2 and Plk1) and dephosphorylations by PP1, PP2A:B55, PP2A:B56 and Cdc14; binding to 14-3-3 proteins, proteolysis and Proline isomerisation [30,31]. For more detail on the regulatory mechanisms involving Wee1 and Cdc25s see Figure 1 and 2. Myt1 regulation also requires further exploration. This kinase contains a C-terminal regulatory domain that is separated by from the kinase domain by a membrane anchor region that targets it to cytoplasmic membrane systems [97]. In Xenopus oocytes Myt1 is subjected to multi-site phosphorylation by p90rsk and also Cdk1/Ringo that interact in a two-step activation mechanism [98,99], but is unclear if a similar regulation occurs in somatic cells.

The precise impact of these regulatory mechanisms on the G2/M transition in mammalian cells remains largely unexplored. Probably the most obvious question concerns the complexity of this system that ultimately results in a simple biochemical dephosphorylation reaction. This emphasises the critical nature of this on/off switch that has to be subjected to extremely fine-tuned control mechanisms to prevent pre-mature or delayed Cdk1 activation. Another surprising feature of this system is that, despite the evolution of these complex control elements, many, if not all, are dispensable for cellular proliferation and even animal development. In one recent example, [100] *et al.* engineered HT2-19 cells that lack the inhibitory phosphorylation sites, Thr14 and Tyr15, in Cdk1. Surprisingly, they found that these cells could proliferate. Likewise, Coudreuse *et al.* [65] found that *S. pombe* can maintain the cell division cycle in the absence of Cdk1 inhibitory phosphorylation. These are extreme examples, but it will be important to analyse, if human cells generally can proliferate in the absence of the entire Wee1/Cdc25 control system, and how other features such as phosphatase control can maintain a functional G2/M transition in the case of constitutively active Cdk1.

There is probably also a direct connection between complexity and robustness in this system. This means that these extremely multi-layered and diverse control mechanisms have evolved as fail-safe mechanisms to generate an extremely robust system that functions with utmost precision in a noisy environment (see below). This is further complicated by differences in tissue specific expression that could introduce subtle but important changes to the G2/M switch in a tissue specific context. A detailed theoretical analysis of this regulatory network will be necessary to fully understand this connection between complexity and robustness. It remains to be established how G2/M network complexity contributes to cellular survival and the prevention of deregulated cell proliferation in multi-cellular organisms.

Releasing the Brake: Phosphatase inactivation during mitotic entry

The initial efforts in understanding cell cycle control of mitotic entry focused primarily on Cdk1 activation. Downregulation of phosphatases, on the other hand, constitutes the flip side of this mechanism, but has only recently become a focus of attention. Early models based on work in Xenopus already suggested a critical role for phosphatase regulation [101-103]. Likewise, the phosphatase inhibitor okadaic acid (OA) has long been known to accelerate mitotic entry, even when Cdk1 is inhibited [104]. It makes sense to imply that Cdk1 antagonising phosphatases are downregulated in mitosis, since activation of Cdk1 against a background of high phosphatase activity results in futile cycles of phosphorylation and dephosphorylation and may prevent the establishment of a stable mitotic state [105,106]. In budding yeast, the phosphatase Cdc14 plays a prominent role in counteracting Cdk1 dependent phosphorylation, but this does not appear to be conserved in other eukaryotes [107]. The discovery of Greatwall kinase [108,109] and its implication in the regulation of PP2A:B55 [110-114] precipitated renewed interest in this area, and recent work from a number of laboratories suggests that phosphatase inactivation plays at least an equally important role than Cdk1 activation in the dynamics of mitotic entry [115]. While PP2A:B55 certainly is a major antagonist of Cdk1, other phosphatases such as PP1, Fcp1, and potentially Cdc14 paralogues are likely to contribute to mitotic entry control in mammalian cells.

Greatwall kinase and PP2A:B55

Greatwall is a kinase of the AGC family that plays a critical role in mitosis in animal cells [116]. Fly embryos lacking this activity fail to establish mitosis and show chromosome condensation defects [108]. Moreover, Greatwall depletion prevents mitotic entry in Xenopus egg extracts [109], where it participates in the Cdk1 auto-amplification loop by downregulating PP2A:B55 [112-114]. Conversely, siRNA depletion of the human Greatwall orthologue, MASTL, [117] or Cre-recombinase induced gene deletion in mouse embryonic fibroblasts [118,119] only shows mild delays in mitotic entry, but causes severe problems during mitotic exit. The only described substrates of Greatwall are two closely related proteins Ensa and ARPP19 [120,121]. Greatwall phosphorylates these small unstructured proteins at S67. This phosphorylation site has strong affinity for the catalytic pocket of the phosphatase PP2A:B55, but is a poor substrate for dephosphorylation. Thus, phosphorylated Ensa/ARPP19 act as PP2A:B55 inhibitors by an unfair competition mechanism [122] by outcompeting the substrates of this phosphatase. Upon inactivation of Greatwall during mitotic exit, the balance shifts towards dephosphorylation causing a slow release of the inhibitor and reactivation of the phosphatase [123].

The role of this pathway during mitotic entry is twofold. Firstly, PP2A:B55 is one of the major Cdk1 antagonising phosphatases and targets many mitotic Cdk1 substrates [124]. Shutting this activity down helps stabilising phosphorylation sites and buffers against potential fluctuations in kinase activity. Secondly, PP2A:B55 is also directly involved in the Cdk1 activation feedback loops by targeting Wee1 and Cdc25 [112-114]. However, as outlined above, there are many layers of

regulation in the switch from Wee1 kinase to Cdc25 phosphatase and we do not know how they are regulated by PP2A:B55. The precise impact of this phosphatase activity on Wee1/Myt1 and the three Cdc25 paralogues in human cells remains to be fully characterised. Moreover, other roles for Greatwall mediated inhibition of PP2A:B55 in S-phase have been recently discovered [125], and mouse genetics suggest that the paralogues Ensa and ARPP19 play surprisingly different roles in development suggesting potential divergent functions [126].

These proposed prominent roles of PP2A:B55 as the major Cdk1 antagonising phosphatase are challenged by the relatively minor mitotic entry phenotypes that have been reported following both Greatwall [117,118] and B55 depletion [123,127] in mammalian cells. Precise measurements of mitotic entry dynamics in B55 depleted cells have not been published, but the available data [123,127] suggest that the effects of siRNA depletion do not compare with the dramatic premature mitosis caused by OA treatment [104]. This could suggest the involvement of other OA sensitive phosphatases such as PP1 in regulation of the G2/M transition, or be a result of insufficient depletion of the various B55 paralogues. More work will be required to further establish which phosphatases are responsible for preventing premature mitosis.

PP1 and PP2A:B56

These two phosphatases are known to play a critical role in providing localised phosphatase activity at the kinetochore to regulate microtubule attachment, SAC signalling and sister chromatid separation [128]. Thus, a fine-tuned balance of various kinase and phosphatase activities is critical for the coordination of mitotic progression. There is a considerable amount of cross talk between these enzymes, and often phosphorylation at one residue recruits a phosphatase to dephosphorylate another residue at the same or a nearby protein. PP2A:B56 also has been implicated in mitotic entry downstream of Chk1 by dephosphorylating Thr130 in Cdc25C, a residue that is phosphorylated by interphase Cdks to promote phosphatase activation [129]. An Ensa/ARPP19 like protein called Bod1 was recency discovered that appears to play a role in tuning the activity of this phosphatase at the kinetochore [130,131], but there is little evidence that, overall, PP2A:B56 is actively downregulated to promote the G2/M transition.

PP1, on the other hand, may play a prominent role in antagonising Cdk1 activation and the phosphorylation events that lead to mitotic entry. This was suggested by a study in Xenopus egg extracts that analysed the effects of PP1 inhibition using recombinant inhibitors [132]. Cdk1 directly downregulates PP1 by phosphorylating the catalytic subunit at a conserved residue Thr320 [133,134], suggesting a feedback loop between kinase activation and PP1 inactivation. Conversely, PP1 also works in concert with Cdk1 to promote Cdc25C activation [135-137]. Moreover, induced expression of the PP1 inhibitor NIPP1 in mammalian cells does not appear to actively promote premature mitotic entry [138].

The precise substrates of the individual mitotic phosphatases and the dependency of substrate specificity on regulatory subunits remains to be explored in depth. A first step in this direction was

recently performed by Cundell *et al.* [124], who analysed the substrates of PP2A:B55 using a combination of genetics, large scale proteomics and modelling. This work suggests that basic residues near the phosphorylation site on the substrate interact with acidic residues at the surface of B55 near the catalytic site of PP2A to direct and stabilise substrate/phosphatase interactions.

There appears to be substantial cross-talk between phosphatases. For example, Grallert *et al.* [139] showed that direct interaction between PP1 and PP2A:B55 results in PP2A:B55 activation. This in turn allows the dephosphorylation of a PP1 docking site in PP2A:B56 and allows the activation of this phosphatase. Likewise, PP1 is important for the inactivation of Greatwall during mitotic exit and dephosphorylates a C-terminal residue in the kinase that is essential for its activity [140-142]. Thus, PP1 indirectly causes PP2A:B55 activation by inactivating Greatwall.

Other Phosphatases

Besides PP1 and PP2A other phosphatase are likely to also contribute to dephosphorylation in mitosis. PP6 has recently been implicated in the downregulation of Aurora kinases [143,144], while mitotic roles for PP4 have been demonstrated in Drosophila [145]. More work will be necessary to determine the overall contribution and regulation of these phosphatases to mitotic entry and progression. Another enzyme that could have an important impact on mitotic dephosphorylation is the RNA polymerase C-terminal domain phosphatase Fcp1. The Grieco lab have proposed that cells lacking this phosphatase delay reactivation of the APC/C and Wee1 kinase during mitotic exit [146,147]. Moreover, our lab reported that depletion of Fcp1 prevents Ensa/ARPP19 dephosphorylation when mitotic exit is triggered by Cdk1 inhibition [148]. This is unlikely to be due to direct dephosphorylation of Ensa/ARPP19 by Fcp1, but it could be a result of delayed Greatwall dephosphorylation in Fcp1 depleted cells [149]. An unresolved question regarding the contribution of Fcp1 to mitotic regulation is its essential role in RNA polymerase II activation. This will need to be resolved by mitotic specific depletion of this phosphatase to avoid indirect effects from changes in transcription throughout interphase.

Finally, the contribution of Cdc14 phosphatases to mitotic progression remains to be fully explored in mammalian cells. Cdc14 is the major Cdk1 antagonising phosphatase in budding yeast, but it's essential functions are not conserved in *S. pombe*, nematodes and flies [107,150]. There are three Cdc14 paralogues in human cells, Cdc14 A, B and C. Phenotypes of cells lacking either Cdc14A and B have been reported involving various aspects of cell cycle control. However, genetic deletion of either Cdc14A and B in cell lines or mice does not appear to affect the G2/M transition or mitotic progression [151]. We do not have data on the effect of complete depletion of all Cdc14 paralogues in human or mouse cells, but given the lack of cell cycle phenotypes in other model organisms it is unlikely that these phosphatases play a dominant role in mitotic regulation.

An important question that remains to be addressed in the balance of kinase and phosphatase activity in mitosis, and indeed many other aspects of cell signalling, is the extent to which these

enzymes are inversely regulated. Shutting down phosphatases while kinase activity is rising may be desirable to stabilise a certain signalling pathway. However, rapid cycles of phosphorylation and dephosphorylation could be an important mechanism to generate fast signalling responses. Thus, the bulk of mitotic Cdk1 phosphorylations are likely to be stabilised by inactivation of the Cdk1 antagonising phosphatases. However, phospho-sites that need to be rapidly removed as soon as Cyclin B levels start to drop in anaphase are likely to be subjected to a tug of war between kinases and phosphatases, and in these cases the relevant phosphatases are expected to be highly active in mitosis. A case for this type of dynamic signalling in mitosis has recently been made [105] and the reader is referred to this excellent review.

Organising mitosis: Open questions in the mitotic entry field

A range of questions regarding the regulation of mitotic entry remain unanswered. Clearly this field lags behind in detailed analysis compared to other areas in cell cycle control. One of the reasons for this is the lack of good synchronisation tools to obtain large numbers of cells in G2 and early M-phase for biochemical and proteomic analysis. However, recent advances in genetic manipulation and also in improved quantitative analysis of single cells have resulted in significant progress, especially concerning studies in human somatic cells. Below, we will give a brief overview on several areas that we consider as crucial questions in mitotic entry. This is a personal choice and there are many other areas of equal importance that we cannot cover within the scope of this review.

Timing of mitotic entry

The most basic task of cell cycle control in eukaryotic cells is to establish a strict temporal separation of DNA synthesis and cell division. A simple form of this control circuit is evident in the rapid embryonic cell cycles where S and M-phase alternate in 30-minute cycles. Most mammalian cells introduce a prolonged gap phase, G2, in between S and M-phase, and the precise timing of the beginning and end of this G2 phase is a critical, yet poorly understood, question in the field. Several recent papers have made significant progress in this area. Saldivar *et al.* carefully analysed the role of ATR in preventing premature mitosis [152]. This revealed that ATR-Chk1 checkpoint kinases constitutively prevent the initiation of a G2 specific wave of transcription as long as replication proceeds. Upon completion of DNA synthesis this inherent S/G2 checkpoint is relieved and Cdk1 phosphorylates G2 specific transcription factors including FOXM1 and B-MYB resulting in the synthesis of proteins involved in the G2/M transition including Cyclin B. Likewise, results from the Lindqvist lab demonstrate a distinctive increase in Cdk1 and Plk1 activity coinciding with the completion of DNA replication [153,154], and demonstrate that DNA replication generates a direct brake on mitotic entry. They establish conditions where origin licensing and firing is suppressed and find that this results in an accelerated mitotic entry in the absence of S-phase. These two papers jointly suggest a model whereby DNA replication generates an intrinsic activation of the ATR/Chk1

pathway that delays the activation of Cdk1 and Plk1 until the genome is duplicated. Once the last stretches of DNA are replicated this brake is relieved, Cdk1 and Plk1 are activated and a G2 transcription program is initiated that results in timely mitotic entry.

This model leaves several questions that remain to be addressed. Firstly, the observation that Cdk1 is already activated at the beginning of G2 phase contradicts the earlier models in which Cdk1 activation occurs in an explosive ultra-sensitive fashion driven by positive feedback at the onset of mitosis [61,136]. This observation also contradicts other measurements of Cdk1 and Plk1 activity with different FRET probes [38,155] and this may be explained by different phosphatases targeting these probes. If Cdk1 is already active in early G2 phase then what prevents the initiation of the feedback loops for another 3 to 4 hours throughout G2 phase? It is unlikely that this G2 delay in triggering full Cdk1 activation depends on further Cyclin B accumulation, since this Cyclin does not appear to be rate limiting for mitotic entry. The complex and multi-faceted regulation of Wee1 and Cdc25 may be critical in generating this time-lag between the first activation of Cdk1 and the initiation of positive feedback loops at the end of G2 phase. Changes in localisation that are likely dependent on specific thresholds in Cdk1 activity could also provide a potential delay mechanism. This has been observed for a variety of G2/M regulators such as Cdc25s, Wee1, Plk1, Cyclin B and Greatwall (reviewed by [7,15,156]).

Another contentious issue in this pathway concerns the role of Plk1. Inhibition of Plk1 by chemical genetics [157], or using a specific small molecule inhibitor [158] delays but does not prevent mitotic entry. Other studies suggests that this is due to incomplete inhibition. Thus, higher and continuous exposure to fresh inhibitor appears to prevent Cdk1 activation and mitosis in a cell type dependent manner [155,159]. Work in Xenopus [160] suggests that the major mechanism of triggering mitosis lies in the Cdk1:CycA dependent activation of Plk1, and in human cells Cyclin A appears to translocate into the cytoplasm in G2 phase to initiate Plk1 activation [161]. This is triggered by phosphorylation of the Plk1 and Aurora-A interactor Bora [162]. Furthermore, the Medema and Fang labs demonstrated that Aurora-A directly phosphorylates Plk1 at its T-loop causing activation and mitotic entry [163,164]. However, neither Aurora-A inhibition in mammalian cells, nor conditional gene deletion in chicken DT40 cells causes a delay in the G2/M transition and prevent Plk1 activation [165,166], suggesting that other means of regulating this kinase in early mitosis could exist.

Overall, the precise roles of the Plk1-Bora-Aurora-A pathway in the G2/M transition need to be further investigated in human cells using improved genetic tools. It is likely that Plk1 plays a major role in Cdk1 activation, but there may well be other ways of triggering the Cdk1 switch in the absence of this kinase given the redundancy in the Cdc25/Wee1 control network. Finally, the inactivation of mitotic phosphatases also plays an important role in determining the length of G2 phase. How this is integrated in the ATR/Chk1 control and how it relates to Plk1 activation and the Wee1/Cdc25 switch is a critical question that remains largely unaddressed. We can conclude that the end of DNA replication sets in motion a series of events leading to mitotic entry, but the precise

timing and choreography of these steps towards the G2/M transition will need to be explored in much greater detail.

Establishing a stable mitotic state

Biochemical work in Xenopus egg extracts firmly established that despite the constant synthesis of Cyclin B throughout the embryonic interphase, Cdk1 activity rises sharply at the end of G2 phase [32]. Based on the positive feedback loops between Cdk1 and Cdc25 and the double negative feedback between Cdk1 and Wee1, Novak and Tyson provided a first theoretical exploration of this switch system [103]. They concluded that this feedback system is sufficient to generate bistability, a term borrowed from the theoretical analysis of engineered switch systems. This model explains how M-phase is a stable steady state insulated by two different Cdk1 thresholds. To enter mitosis Cdk1 needs to rise above a high entry threshold that is established by the positive and double negative feedback loops between its activators and inhibitors. Once this threshold has been crossed and mitosis is established, Cdk1 activity can drop considerably before mitotic exit is triggered. Predictions from this bistability model include hysteresis (i.e. different responses to changes in Cdk1 levels dependent on the previous state of the system), increasing delays in mitotic entry if Cdk1 activity drops to levels close to the entry threshold, and a spread of Cdk1 activation in space in the form of a trigger wave. Each of these predictions was confirmed experimentally in Xenopus egg extracts supporting the validity of the Novak-Tyson model [167-169]. A similar bistable response was later proposed to be present in human cells [170]. This study suggests that the stabilisation of the mitotic state following Cdk1 activation is critical during nuclear envelope breakdown, when the concentration of cytoplasmic Cdk1:Cyclin B drops significantly.

A major question that remained unanswered by the original bistability model concerned the actual importance of the Wee1/Cdc25 regulation and the parallel influence of phosphatase regulation. The discovery of Greatwall and its function in downregulating the Cdk1 antagonising phosphatase PP2A:B55 challenged the idea that the Cdk1 activation feedback loop is solely responsible for the establishment of bistability in mitotic entry. In vitro work by Mochida *et al.*, subsequently confirmed that the Greatwall PP2A:B55 regulatory circuit itself was a bistable system [171]. Concomitant with this, Rata *et al.* showed that Cdk1 activation and PP2A inactivation are indeed interlinked, yet separate bistable switches in human cells [106]. This study concludes that only loss of both control systems eliminates bistability.

An interesting prediction from the model presented by Rata *et al.* was the presence of a theoretical new steady state in prophase. This steady state only exists if Cdk1 activity and PP2A:B55 are balanced and remain locked in an intermediate state of substrate phosphorylation and dephosphorylation. Indeed, this could be experimentally verified by inhibiting Cdk1 in prophase cells to a level at which it could not overcome its counteracting phosphatases to reach NEBD and M-phase, but also not drop sufficiently to cause reversion to G2-phase [106]. This prophase state is avoided under normal circumstances when Cdk1 activity rises sharply and quickly overcomes

PP2A:B55 with the help of Greatwall.

Having an established quantitative assay for hysteresis in mammalian cells will be useful to assess other features of this switch system in detail. What is the impact of the different redundant elements such as the Cdc25 paralogues, Wee1 and Myt1? Which phosphatases are important for the G2/M switch? How does the switch system differ among different cell lines, and has it been altered in cancer cells? A critical open question here concerns the cross-talk between the Wee1/Cdc25 switch and PP2A:B55. The model used by Rata *et al.* [106] is based on the assumption that PP2A:B55 directly regulates Cdk1 phosphorylation in this feedback system, and this is supported by results from Xenopus egg extracts [112-114]. However, as Figure 1 and 2 show, we do not have direct evidence that PP2A:B55 indeed regulates some of the many phospho-sites in mammalian Cdc25 phosphatases and Wee1/Myt1 kinases. Ultimately, this theoretical and quantitative exploration of the mitotic entry network will help uncovering its basic design features and may help in designing therapeutic strategies to target this cell cycle transition in clinical applications.

Timing Cdk1 dependent substrate phosphorylation

The bistability model suggests that the feedback driven, switch-like activation of Cdk1 in late G2 phase separates two stable states, interphase and mitosis with a rapid trajectory between them [103]. This successfully describes how Cdk1 activation occurs, but does not explain the fine-tuned stepwise progression that we observe during mitotic entry. If Cdk1 is either off or on, a downstream mechanism must be responsible to switch its substrates from unphosphorylated to phosphorylated in the observed sequential manner. A simple mechanism could involve different catalytic rates of Cdk1 for early (kcat high) and late (kcat low) substrates. This was suggested by work from Gavet & Pines [38], who monitored Cdk1 activity in live cells using a FRET probe, and correlated early events of mitosis (cell rounding, centrosome separation) with low FRET signal, while NEBD only occurred after the FRET signal reached its peak. A similar observation was also made earlier based on quantitative immuno-fluorescence measurements [170]. This threshold mechanism could be further complicated by differing substrate specificities for the earlier Cdk1:Cyclin A and later Cdk1:Cyclin B.

Another potential contributor to introduce time control in the mitotic switch system could be Cdk1's antagonising phosphatase PP2A:B55. The double negative feedback loop between Cdk1 and PP2A:B55 that we described above, ensures that phosphatase activity decreases, as Cdk1 activity increases. One possibility is that early prophase substrates that cause centrosome separation, cell rounding and chromosome condensation are poor PP2A:B55 substrates and become phosphorylated as soon as the initial pool of mitotic Cdk1 is active. Subsequent events could then be triggered, once PP2A:B55 itself is inactivated. A similar mechanism of different catalytic activity of PP2A:B55 towards early and late substrates has been demonstrated to control the timing of cell cycle transition in yeast [172] and during mitotic exit [124,173]. However, mitotic entry and exit are not mere mirror images of the same sequence of events but involve markedly different

processes. It remains to be seen if, and to what extent catalytic rates of Cdk1:Cyclin A, Cdk1:Cyclin B and PP2A:B55 contribute to the establishment of temporal order during mitotic progression in human cells.

The specific subcellular localisation and sequestration of the various kinases, phosphatases and other regulatory proteins that contribute to the Cdk1 activation feedback loops constitutes another critical aspect of this switch system. This is likely to promote robust timing of mitotic events (reviewed by [7,15]). A comprehensive numerical model of mitotic entry should incorporate both spatial and temporal regulation to fully capture these important features of mitotic control.

Robustness of the mitotic switch

In this review we have outlined some of the complexity that underlies the control of Cdk1 dependent phosphorylation events at the onset of mitosis. Robustness [174,175] is a likely reason for the evolution of this complexity that ultimately controls a relatively simple outcome, namely the activation of a single kinase. This means that despite the variability of the environment and the considerable molecular noise in this system, mitosis has to be triggered at exactly the right time and, once triggered, proceed fast and unidirectionally. There are two aspects to be considered here: Firstly, as discussed above, robustness of mitotic establishment is likely to depend on the positive feedback loops that generate the bistable switch system [176]. The linking of Cdk1 activation and phosphatase inactivation (Figure 3) provides a network architecture that ensures a robust transition between interphase and mitosis with maximum theoretical efficiency [177]. Simultaneously, robustness in the timing of mitotic entry relies on this ultra-sensitive switch to be highly resistant to noise, because once it is triggered there is no way back. This is a general problem of biological systems that rely on positive feedback. One could say that the cell has to be ready for explosive reactions, but simultaneously prevent these explosions to happen at the wrong time. The multiple combinations of possible inputs in this network, as exemplified by the various regulatory elements in Wee1 and Cdc25 control, may serve as a buffer that maintain the off state in a noisy environment. At the same time, there are likely to be vulnerable core reactions that need to be tightly controlled to prevent catastrophic misregulation due to fluctuations in protein levels or activity. This is exemplified by the dramatic effects of okadaic acid that rapidly triggers cellular changes typical for mitosis in cells with low Cdk activity. This suggests that the maintenance of high phosphatase activity is one of the potential vulnerable links in this network that does not tolerate large fluctuations. A detailed sensitivity analysis of the mitotic switch system will be necessary to uncover the basic design features and identify the most vulnerable elements in the control of mitosis. An interesting idea that has been put forward by [178] proposes that coherent feed forward loops are responsible for dampening signal fluctuations and prevent premature activation of the positive feedback loops that cause Cdk1 activation and mitotic entry. This type of suppression of short signalling pulses and persistence filtering by coherent feed forward loops has been explored in the regulation of transcription [179]and may well apply to the robustness of the

G2/M switch. PP2A:B55 inhibition by Greatwall downstream of Cdk1 can be interpreted as a coherent feedforward loop [178]. However, siRNA depletion of PP2A:B55 does not appear to dramatically change the dynamics of mitotic entry [123,127], suggesting that other pathways may also prevent noisy and fluctuating input signals from triggering Cdk1 activation.

Mitotic entry and cancer

Even though the core cell cycle engine is highly conserved, cancer cells show many alterations in cell cycle signalling pathways. Consequently, cell cycle control remains one of the prime targets for successful cancer therapy [180]. In this context, the G2/M transition is becoming an area of renewed interest as a target in cancer therapy. This is exemplified by the recent success of new targeted therapies involving Wee1 inhibitors [10]. Likewise, Greatwall kinase has been proposed to be a promising target in a variety of cancer types including breast, lung and ovarian cancer [181-186]. It appears that deregulation of the Greatwall/PP2A:B55 axis is a crucial alteration in many types of tumours. High expression of Greatwall has been associated with poor prognosis in triple negative breast cancers and the PP2A regulator B55-alpha is deleted in 15% of prostate cancers [187]. Current models suggest that PP2A:B55 acts as a tumour suppressor and cancer cells often employ high levels of Greatwall to down-regulate this anti-proliferative phosphatase activity [11]. This has been suggested to sensitise cells to loss of Greatwall activity. However, potent and specific inhibitors to this kinase are not yet available to test this hypothesis.

One could speculate, given the potential for Wee1 and also Greatwall inhibition in targeted therapy, that certain tumour cells have altered the core Cdk1 activation network. Such changes could explain why untransformed glial stem cells are significantly less sensitive to Wee1 inhibition compared to aggressive glioma cancer cells [188], or why some triple negative breast cancer cells are highly sensitive to Greatwall depletion while others can proliferate with very little Greatwall activity [182]. It remains to be determined why and how the G2/M switch system has been altered in these sensitive cancer types. One possibility that remains to be explored is that elevated levels of replication stress necessitate changes in the regulation of Cdk1 activation. This implies that certain cancers have decreased the robust mitotic entry block in response to DNA damage to accommodate increased levels of stress signals from unscheduled replication. One way of achieving this could be by downregulating PP2A:B55 or Wee1 activity, thereby exposing the tumours to specific fragilities in these pathways. This is, of course, highly speculative, and other possibilities such as G2/M independent functions of Greatwall, PP2A:B55 or Wee1 may also explain the observed sensitive responses to Wee1 inhibitors and Greatwall depletion.

The important next steps for the development of this field towards clinical application are a more detailed mechanistic understanding of changes in the G2/M network and subsequently the identification of predictive biomarkers that could inform clinical applications. To this end it will be necessary to compare the G2/M switch system in non-transformed cells with tumour cells that are highly sensitive to Greatwall or Wee1 inhibition based on quantitative measurements and

mathematical modelling. A better understanding of the underlying changes that result in the observed sensitisation may help developing diagnostic tools to predict which cancers are amenable to treatment using Wee1 and future Greatwall inhibitors. Moreover, further analysis of the G2/M network and its links to metabolism, DNA damage response and size control may also reveal additional therapeutic targets. Overall, we are likely to see interesting developments in this area of cell cycle control in the coming years.

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Figure 1 Regulation of Wee1

Model summarising some of the key steps in Wee1 regulation. We used green fonts for activating steps and orange fonts for inhibitory modifications. **1)** After its synthesis in the cytoplasm, Wee1 rapidly binds to its chaperone HSP90 and promotes Tyr38 phosphorylation in HSP90. This may contribute to Wee1 stabilisation and nuclear import. [189-191]. **2)** Throughout interphase, nuclear Wee1 is phosphorylated by Chk1 on Thr642, promoting the binding of 14-3-3 isoforms to the C-terminus. This is promoting Wee1 activity and may also support its nuclear retention. [192-195] . **3)** In contrast, Ser642 phosphorylation by AKT kinase promotes cytoplasmic localisation of Wee1 upon onset of mitosis due to binding to distinct isoforms of 14-3-3 [196]. Accordingly, phosphorylation of the Xenopus equivalent of Ser642 (Ser549) appears to peak during mitosis [197]. It is possible that two different sets of 14-3-3 isoforms are able to influence the same site and are likely regulated in some other unknown way. More work will be required to shed light on the activation and deactivation of Wee1 by phosphorylation and 14-3-3 binding. **4)** Inactivation of Wee1 at the onset of mitosis is initiated by Cdk1 dependent phosphorylation of Ser123. This promotes nuclear export and destabilisation of Wee1 [198-201]. Thus, the main switch between active and inactive Wee1 appears to occur via removal of 14-3-3 binding and dephosphorylation of Thr642 and phosphorylation by Cdk1. The phosphatase that targets Thr642 is thus likely to be critical but remains unknown. The Cdk1 site Ser123 appears to be dephosphorylated in the cytoplasm by Cdc14A [202], but the relevance for this activity in cell cycle control is unclear given the unchanged mitotic entry dynamics in Cdc14A knock-out cells [151]. Moreover, it remains unclear to what extent Akt kinase dependent phosphorylation and potentially a switch in 14-3-3 isoform may contribute to Wee1 inactivation. **5)** The phosphorylated Ser123 residue acts as a docking site for Plk1 and Ck1. These two kinases phosphorylate Ser121 (Ck1) and Ser53 (Plk1) and further enhance destabilisation of Wee1 [200,201,203]. Two forms of the SCF ubiquitin ligase SCF BetaTRCP [200] and SCF-Tome1 [204] contribute to Wee1 ubiquitylation and destruction during mitotic onset. The model shown above only refers to a limited number of important steps in Wee1 regulation. There are many other regulatory events that contribute to Wee1 activity such as autophosphorylation (Tyr295 and Tyr362) [196], Proline isomerisation by Pin1 [205], and interaction with the MIG6 tumour suppressor [206]. More work will be necessary to investigate the interplay of these post translational modifications and their relevance to the dynamic regulation of mitotic entry.

Figure 2 Regulation of Cdc25

Model summarising some of the key steps in Cdc25 regulation. We used green fonts for activating steps and orange fonts for inhibitory modifications. 1) Mouse genetics suggest that Cdc25A is essential for Cdk activation in early development [95]. Accordingly, Cdc25A has roles both in G1/S [207,208] and G2/M transitions where it seems to coordinate chromosome condensation [209]. Moreover, it is often overexpressed in cancerous tumours [210], suggesting that its over-expression could contribute deregulation of the cell cycle. Deletion of this phosphatase in somatic tissues and mouse ES cells, on the other hand, does not prevent proliferation, suggesting that either Cdc25B, or C can compensate for its functions under these circumstances [94-96]. The regulation of this protein involves proteasomal degradation at different times of the cell cycle and part of the Cdc25A activation mechanisms involves preventing its destruction. Its common overexpression in cancers demonstrates the need for a highly complex temporal control of the Cdc25A phosphatase activity. A key step in Cdc25A regulation involves its SCF Beta-TrCP dependent proteasomal degradation. This is triggered via Chk1 or Chk2 phosphorylation on Ser123 in the presence of DNA damage during S and G2 [211-215]. Additionally, Cdc25A can be downregulated to delay G1/S transition via a negative feedback loop involving Cdk4/6:CycD [216]. Moreover, it is also targeted by the APC/C in late mitosis and G1 [217] to prevent Cdk1 reactivation and delay entry into S-phase. Concomitant with this, when the G1 or G2 checkpoints are triggered, this protein is rapidly degraded to prevent Cdk1 and cell cycle progression and allow time for DNA repair [211,214]. Upon the onset of mitosis, Cdk1 contributes to Cdc25A stabilisation by Ser17 and Ser115 phosphorylations [214]. However, this may not be the only mechanism, but the contribution of other mitotic kinases such as Plk1 to Cdc25A regulation remains unclear. During mitotic entry Cdc25A is also regulated by 14-3-3 binding, where Ser178 acts as a docking site. Both Chk1 and Chk2 can phosphorylate Ser178 and at least Chk1 can also phosphorylate Thr507 to aid 14-3-3 binding which inhibits Cdc25A activity [218,219]). This is thought to further delay premature mitotic entry.

2) Cdc25B also contributes to the G2/M transition and likely activates Cdk1/CycB at centrosomes [220-222]. Cdc25B is also often overexpressed in cancer and this can cause premature mitotic entry and replication stress [210,223]. Moreover, Cdc25B has been implicated to have an additional function besides its phosphatase activity in aiding the activation of steroid receptors, which could contribute to its role in promoting cancerous tumours when overexpressed [224,225]. It is a likely scenario that Cdc25B initiates cytoplasmic Cdk1/Cyclin B activation which then aids in activating Cdc25C to ensure a full activation of the mitotic kinase [226]. Therefore, it is essential that Cdc25B is kept inactive in interphase. This is achieved by Chk1 that phosphorylates Ser230 even in the

absence of DNA damage [227], likely to avoid a premature mitotic entry. The localisation of Cdc25B is important for its function and the protein contains both an NLS (aa 335-353) and two NES sequences (aa28-40; aa 55-67) [228,229]. Similar to Cdc25C, 14-3-3 protein binding also aids in regulating Cdc25B localisation [228,230]. However, it is not entirely clear how 14-3-3 binding is regulated and how it precisely affects NES and NLS activity. Dissociation of Cdc25B from 14-3-3 proteins contributes to its activation by allowing access to its catalytic and NES domains [231]. It may simultaneously also affect nuclear import and increase overall shuttling rates. Another important step in Cdc25B activation is the phosphorylation on Ser353 by [232,233] and this appears to be regulated by the DNA damage response via Chk1 [232,234]. **3)** Cdc25C is kept inactive during interphase via at least two phospho-sites, Ser 216 and Thr130. Ser216 phosphorylation by variety of kinases including Chk1/2, C-TAK, CaMKII and PKA [235-237] promotes binding of 14-3-3 protein that aids in suppressing Cdc25C and sequestering Cdc25C in the cytoplasm to avoid premature mitotic entry [238-240]. Thr130 on the other hand, is phosphorylated by interphase Cdk activity that is predominantly CycA:Cdk2. This promotes the dissociation of 14-3-3 proteins and subsequent activation via PP1 dependent dephosphorylation of Ser216 [137,241,242]. The activating Cdk1 site Thr130 on the other hand, is actively dephosphorylated by PP2A:B5. and this is promoted by Chk1 during replication [129]. This tug of war between Chk1/PP2A:B56 and Cdk1/PP1 ensures the correct timing of the activating phosphorylation. Once Thr130 is phosphorylated it generates a docking site for Plk1 [243,244], which aids in nuclear localisation of the protein as well as its nuclear retention by phosphorylating and inactivating a nuclear export site Ser198 [242,245]. Furthermore CK2 contributes to nuclear import via phosphorylation of Thr263[246]. The active state of Cdc25C in the nucleus is further stabilised by Cdk1 dependent Ser214 phosphorylation [135,136] and this further strengthens the association with PP1 [137], thereby reducing the chance of Chk1 mediated inactivation. Pin1 dependent Prolyl-isomerisation induced by phosphorylation has also been suggested to play an important role in Cdc25C regulation in Xenopus [247,248], but the impact of this pathway in human cells requires further exploration.

Figure 3 Robust control of Cdk1 activation by two interlinked bistable switches

A body of evidence suggests that Cdk1 in complex with Cyclin A constitutes the trigger of mitotic entry [70-73]. Our recent work using degron tags provided further evidence that this is, indeed a G2-specific function of Cyclin A [78]. The action of this trigger is twofold: Firstly it sets in motion the feedback loops that lead to ultra-sensitive and bistable Cdk1 Try15 dephosphorylation by Cdc25 activation and Wee1 inhibition [103,136,167,168] and secondly it triggers inactivation of PP2A:B55 via activation of Greatwall and phosphorylation of Ensa/ARPP19 that also follows the dynamic of a bistable system [171]. Our model also suggests that these two bistable switch systems interlink [106], although the precise biochemical mechanisms of this crosstalk remain to be established. A theoretical analysis of this type of interlinked bistable switch suggests that this operates with maximal theoretical stability and efficiency [177]. This model raises further questions that remain to be addressed: Why can Cyclin B not replace Cyclin A as the trigger? How does it involve other phosphatases such as PP1, Cdc14 or Fcp1? To what extent does this switch depend on Plk1 activity? What determines the precise timing of this activation switch? How has it been changed in cancer cells to generate increased sensitivity to Wee1 and Greatwall inactivation?

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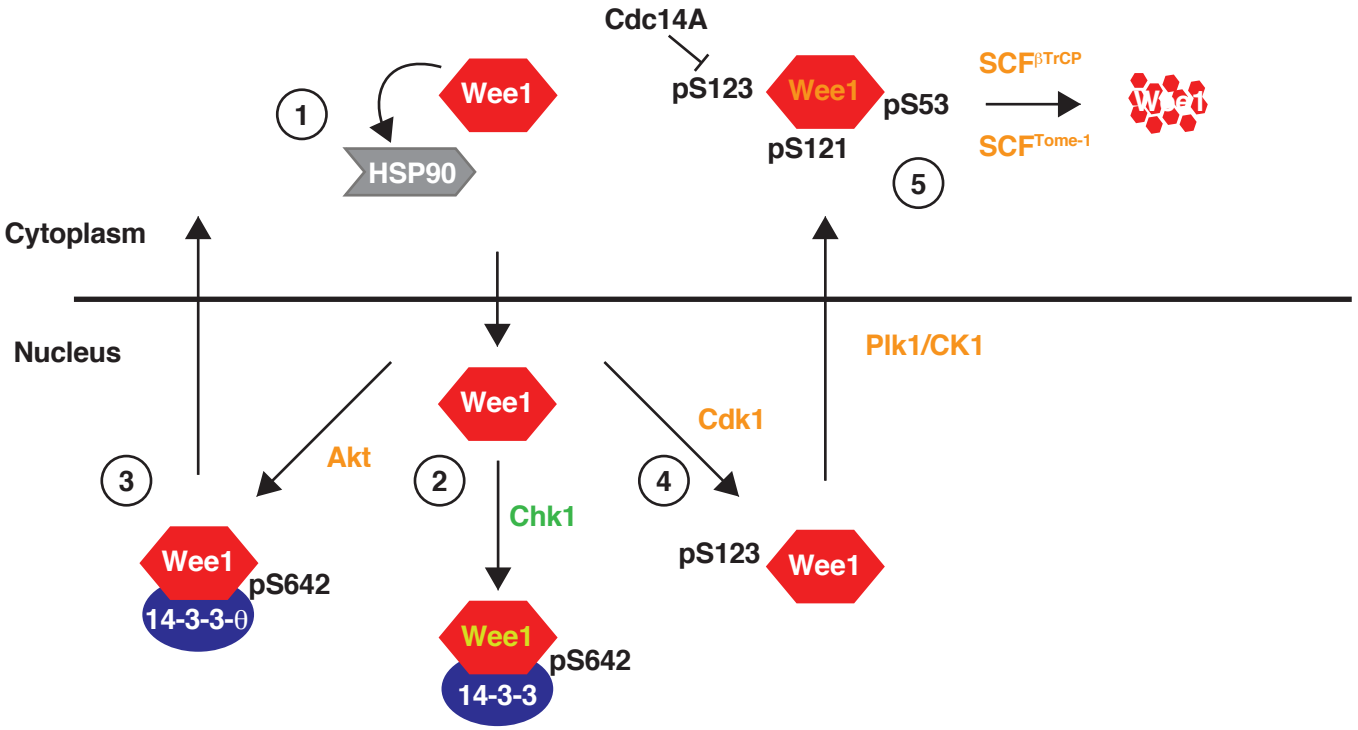
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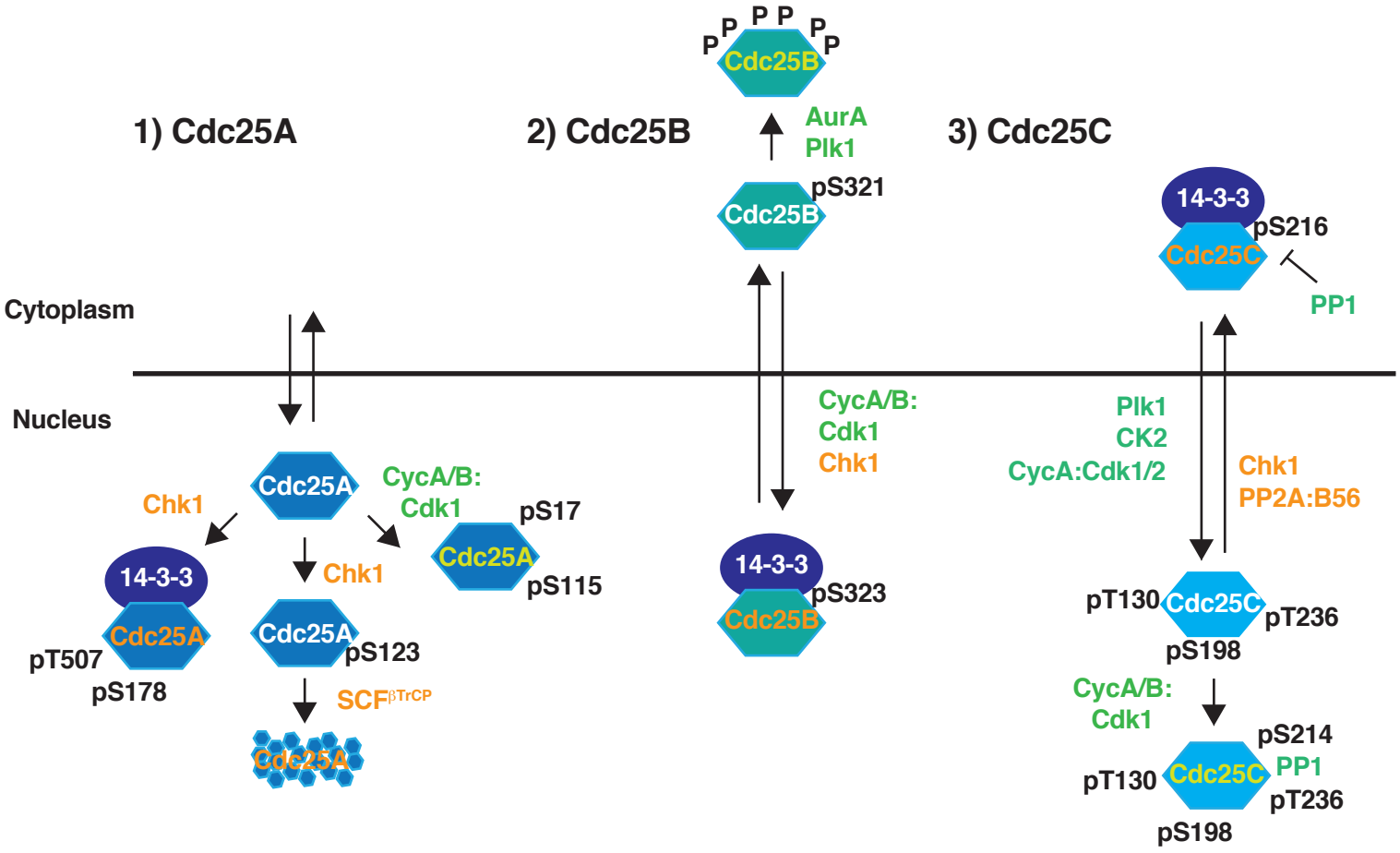
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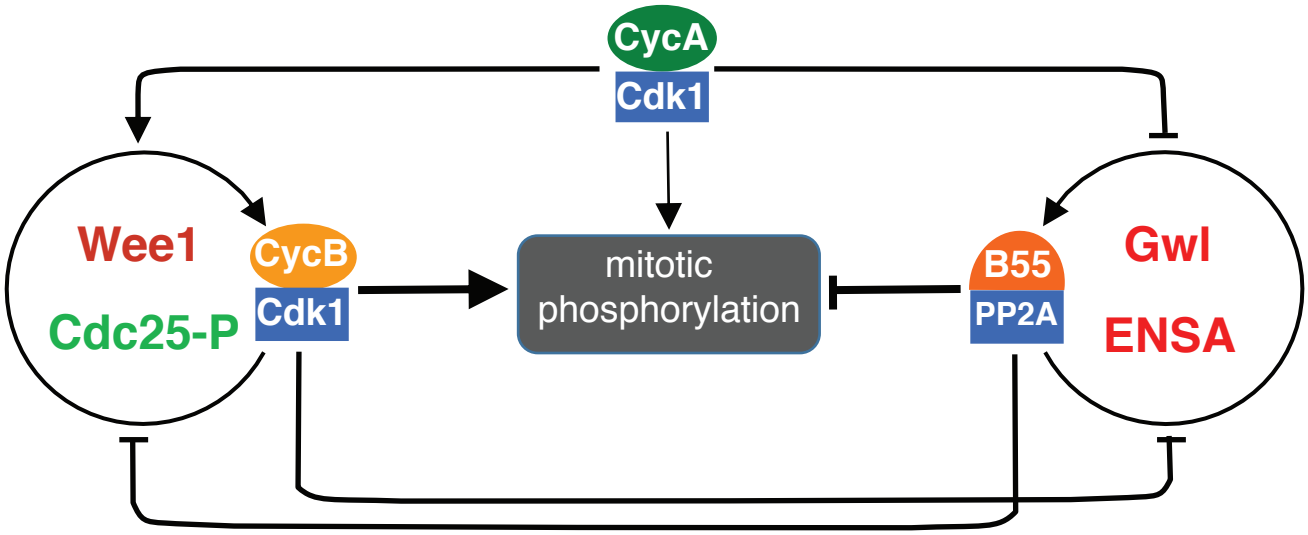
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